Express Mail No.: EL737854633US
BD Ref. No.: P-5677

Neisseria Gonorrhoeae Assay

FIELD OF INVENTION

[001] The present invention relates to nucleic acid amplification methods for the detection and/or quantitation of nucleic acid sequences of *Neisseria gonorrhoeae*. The present invention provides oligonucleotides that are complementary or that anneal to nucleic acid sequences of *Neisseria gonorrhoeae* for the amplification and/or detection of the same. The present invention also provides internal amplification controls (IACs) that can be used in nucleic acid amplification reactions to determine whether the assay conditions are permissible for amplification and/or detection of a target sequence. The present invention provides a diplex strand displacement amplification (SDA) assay for the amplification and/or detection of *Neisseria gonorrhoeae* nucleic acid sequences in the presence of an IAC.

BACKGROUND OF THE INVENTION

[002] Gonorrhea is the most prevalent communicable disease reported in the United States, with an estimated 2.5 million or more cases reported annually. *See* Tierney *et al.*, Current Medical Diagnosis and Treatment, 37th ed., 1998, Appleton & Lange. Gonorrhea is caused by *Neisseria gonorrhoeae*, a gram-negative diplococcus bacterium typically found inside polymorphonuclear cells, and is most commonly transmitted during sexual intercourse. These bacteria can infect the genital tract, the mouth and the rectum. In women, the opening to the uterus, the cervix, is the first site of infection. The incubation period of the bacterium is usually 2-8 days (*see* Tierney *et al.*, Current Medical Diagnosis and Treatment, 37th ed., 1998, Appleton & Lange). Gonorrhea is an important cause of urethritis in men and cervicitis in women. Gonorrhea may spread into the uterus and fallopian tubes resulting in pelvic inflammatory disease ("PID") and in fact approximately 20% to 40% of PID and 14% of tubal infertility can be attributed to gonococcal infections. *See* Chan *et al.*, 2000, Arch. Pathol. Lab. Med. 124:1649-1652.

[003] Traditional laboratory diagnosis of gonorrhea is done by an overnight culture of clinical swabs (*e.g.*, urine or cervical) obtained from a subject followed by biochemical and/or microscopic identification of *Neisseria gonorrhoeae*.

[004] Recently, nucleic acid amplification tests have become widely used for detection and/or diagnosis. Currently available commercial *Neisseria gonorrhoeae* DNA amplification tests include PCR (Roche Molecular Systems, Branchburg, NJ), and strand displacement amplification (SDA; Becton Dickinson, Sparks, MD). *In vitro* nucleic acid amplification techniques provide powerful tools for detection and analysis of nucleic acids, especially when the target nucleic acids are present in small quantities. The sensitivity of such methods has made them particularly suitable in areas such as medical diagnosis (*e.g.*, detection of infectious agents like bacteria and viruses, diagnosis of inherited and acquired genetic diseases, and the establishment of tissue type), isolation of genes, and forensic medicine (*e.g.*, forensic tests for detection of specific nucleic acids in criminal investigations).

[005] Nucleic acid amplification techniques are traditionally classified according to the temperature requirements of the amplification process. Isothermal amplifications are conducted at a constant temperature, in contrast to amplifications that require cycling between high and low temperatures. Examples of isothermal amplification techniques are: Strand Displacement Amplification (SDA; Walker *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:392-396; Walker *et al.*, 1992, Nuc. Acids. Res. 20:1691-1696; and EP 0 497 272, all of which are incorporated herein by reference), self-sustained sequence replication (3SR; Guatelli *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), the Qβ replicase system (Lizardi *et al.*, 1988, BioTechnology 6:1197-1202), and the techniques disclosed in WO 90/10064 and WO 91/03573. Examples of techniques that require temperature cycling are: polymerase chain reaction (PCR; Saiki *et al.*, 1985, Science 230:1350-1354), ligase chain reaction (LCR; Wu *et al.*, 1989, Genomics 4:560-569; Barringer *et al.*, 1990, Gene 89:117-122; Barany, 1991, Proc. Natl. Acad. Sci. USA 88:189-193), transcription-based amplification (Kwoh *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:1173-1177) and restriction amplification (U.S. Pat. No. 5,102,784).

[006] The currently available nucleic acid amplification tests for *Neisseria* gonorrhoea, however, lack an internal control mechanism to assay for any inhibitory reaction conditions or human errors that are present in the tests and are thus prone to false negative results. Thus, there is a need for an assay that decreases the possibility of false negative results.

[007] *Neisseria gonorrhoeae* shares a high degree of homology with other closely related *Neisseria* species. Thus, there is clearly a need for the development of new methods

and oligonucleotides that are able to confirm the results of existing assays and/or increase the specificity and/or sensitivity of a test to detect *Neisseria gonorrhoeae*.

[008] The present invention provides a diplex nucleic acid amplification assay which can be used as an alternative to the current monoplex *Neisseria gonorrhoeae* assay (*i.e.*, no internal amplification control in the same reaction mixture with a target sequence). The present invention also provides oligonucleotides that can be used in both diplex and monoplex nucleic acid amplification assays designed to amplify and/or detect *Neisseria gonorrhoeae* nucleic acids.

[009] Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

SUMMARY OF THE INVENTION.

[010] The present invention provides a method for detecting qualitatively and/or quantitatively the presence or absence of *Neisseria gonorrhoeae* in a sample, said method comprising: (a) amplifying the target sequence using a first amplification primer having a sequence consisting essentially of the target binding sequence of any amplification primer disclosed herein and (b) detecting the amplified target sequence. The present invention also comprises the use of a second amplification primer consisting essentially of the target binding sequence of any amplification primer disclosed herein.

[011] The present invention also provides Internal Amplification Control sequences in order to decrease the occurrence of false negative results.

[012] The present invention also provides a method for detecting a *Neisseria gonorrhoeae* target sequence comprising: (a) hybridizing one or more amplification primers disclosed herein to a target sequence and (b) detecting said hybridized amplification primer.

[013] The present invention also provides oligonucleotides which are useful in the amplification and detection of *Neisseria gonorrhoeae*. The present invention also provides kits comprising such oligonucleotides

BRIEF DESCRIPTION OF THE FIGURES

[014] Figure 1. The positions of the *Neisseria gonorrhoeae* genome to which the amplification primers, bumper primers and adapter oligonucleotides anneal.

DETAILED DESCRIPTION OF THE INVENTION

[015] Any definitions provided are for reason of clarity and should not be considered as limiting. Except where noted, the technical and scientific terms used herein are intended to have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

[016] The present invention relates to nucleic acid amplification methods and assays for the detection and/or quantitation of nucleic acid sequences of *Neisseria gonorrhoeae*. The present invention provides one or more oligonucleotides that are complementary or that anneal to nucleic acid sequences of *Neisseria gonorrhoeae* for the amplification and/or detection of said sequences. The present invention further provides an internal amplification control (IAC) that can be used in nucleic acid amplification assays of the invention to determine whether the assay conditions are permissible for amplification and/or detection of a target sequence. The oligonucleotides may be used in all types of amplification reactions such as, for example, Strand Displacement Amplification (SDA), Polymerase Chain Reaction (PCR), Ligase Chain Reaction, Nucleic Acid Sequence Based Amplification (NASBA), Rolling Circle Amplification (RCA), Transcription Mediated Amplification (TMA) and QB Replicase-mediated amplification.

[017] The methods of the invention are particularly advantageous over traditional methods used for the detection of gonorrhoeae, as they reduce false negative results by, for example, inclusion of internal amplification controls.

[018] Sensitivity of an assay relates to the tolerance of false negatives. A test result is false negative if the test shows negative but the sample actually contains the target sequence. The smaller amount the target sequence an assay can detect, the higher sensitivity an assay has.

[019] Specificity of an assay relates to the tolerance of false positives. A test result is false positive if the test shows positive but the sample actually does not contain the target sequence. Thus, a more specific an assay should have lower level of false positives.

[020] In accordance with the present invention, a result of an assay to detect for *Neisseria gonorrhoeae* in a sample that utilizes an IAC can be interpreted as described in Table 1.

Table 1. Interpretation of a diplex SDA assay

	Result			
IAC	+	–	+	–
Target Sequence for *Neisseria gonorrhoeae*	–	+	+	–
Presence or absence of *Neisseria gonorrhoeae*	absence	presence	presence	inhibitory reaction, assay need to be re-performed or modified

[021] In accordance with the present invention, an IAC may be used instead of, and/or in addition to, a conventional amplification control (AC). It is understood by one skilled in the art that the conventional AC reaction is performed in a separate reaction mixture from the sample to be tested. A conventional AC reaction comprises amplification reagents and target DNA. If the amplification and/or detection of the target DNA in the AC reaction is suppressed, an indication that the target sequence is absent from a test sample may be attributed to inhibitory signals in the reaction. While this form of control reaction is effective, it is not the most desirable. Since the AC reaction is performed separately, it cannot exactly reflect the conditions of the reactions containing the test sample. The methods of the invention are particularly useful in that they have an IAC and the control reaction is performed under identical spatial and temporal conditions as the amplification and/or detection of the target sequence thereby minimizing human error.

[022] The present invention also provides amplification primers that anneal to both a target sequence (*i.e.*, a sequence of *Neisseria gonorrhoeae*) and/or an IAC sequence. In some embodiments of the invention a bumper primer or adapter oligonucleotide or its respective target binding sequence described in Table 2, Table 3 or Figure 1 may be used as an amplification primer. In some embodiments of the invention, an amplification primer is chosen from the amplification primers described in Table 2, Table 3 or Figure 1 as disclosed herein. In another embodiment of the invention, an amplification primer is chosen from the target binding sequences of amplification primers described in Table 2, Table 3 or Figure 1 as disclosed herein. In another embodiment of the invention, the amplification primers comprise GCINT3-APL2 (SEQ ID NO:1) and GCINT3-APR1 (SEQ ID NO: 2).

[023] The present invention further provides adapter oligonucleotides and detection probes that can be used in a nucleic acid amplification assay for the detection of nucleic acid sequences of *Neisseria gonorrhoeae*. In some embodiments of the invention, the adapter oligonucleotide is a single-stranded oligonucleotide comprising SEQ ID NO:5, 6, 18, 20 or 21. In other embodiments of the invention, the detection probe is a single-stranded oligonucleotide comprising SEQ ID NO: 7, 8, 22 or 23, and a fluorescent donor/quencher pair linked to the oligonucleotide.

Amplification Methods

[024] The oligonucleotides disclosed herein can be used in any method of nucleic acid amplification known in the art.

[025] Suitable amplification methods include, but are not limited to, Polymerase Chain Reaction ("PCR"; *see* U.S. Patents No. 4,683,195; 4,683,202; 4,800,159; and 4,965,188), Strand Displacement Amplification ("SDA"; *see* Walker *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:392 (1992); Walker *et al.*, *Nucl. Acids Res.* 20:1691 (1992); and U.S. Patent No. 5,270,184, the disclosure of which is hereby incorporated in its entirety by reference), thermophilic Strand Displacement Amplification ("tSDA"; *see* EP 0 684 315), Self-Sustained Sequence Replication ("3SR"; *see* Guatelli *et al.*, *Proc. Nat'l Acad. Sci. USA* 87:1874-78 (1990)), Nucleic Acid Sequence-Based Amplification ("NASBA"; *see* U.S. Patent No. 5,130,238), Qβ replicase system (*see* Lizardi *et al.*, *BioTechnology* 6:1197 (1988)); Ligase Chain Reaction ("LCR"; *see* U.S. Patent No. 5,427,930); Rolling Circle Amplification (*see* Lizardi *et al.*, *Nat Genet* 19:225-232 (1998)) and transcription based amplification (*see* Kwoh *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:1173-77 (1989)). The amplification primers of the present invention may be used to carry out PCR, SDA or tSDA..

[026] SDA generally proceeds along the following pathway. First, amplification primers bind to a target sequence or to a displaced single-stranded extension product that has been previously polymerized. Second, a 5'-3' exonuclease-deficient polymerase incorporates an α-thiodeoxynucleoside triphosphate ("α-thio dNTP") into an extension product. If the α-thio dNTP is α-thio dCTP, for example, it is incorporated into the extension product wherever there is a complementary G residue in the template. Incorporation of an α-thio dNTP into the extension product at a restriction endonuclease recognition site creates a hemimodified site, *i.e.* a site modified only on the extension product strand. A restriction endonuclease then nicks the hemimodified double-stranded restriction site. Next, the restriction endonuclease dissociates from the nick site. Finally, a polymerase that is deficient

in 5'-3' exonuclease activity extends from the 3' end of the nick and displaces the downstream strand of DNA. Nicking, strand extension and strand displacement occur concurrently and continuously because extension from the nick regenerates another nickable restriction site. When a pair of amplification primers is used that each hybridize to one of the two strands of a double-stranded duplex comprising a target sequence, amplification is exponential because both the sense and antisense strands serve as templates in each round of amplification. When a single amplification primer is used, amplification is linear because only one strand serves as a template for primer extension. Examples of restriction endonucleases that nick their double-stranded recognition sites when an α-thio dNTP is incorporated and that are suitable for SDA include *Bso*B1, *Bsr*I, *Bst*NI, *Bsm*AI, *Bst*OI, *Bsl*I, *Ava*I, *Hinc*II and *Nci*I. SDA is further described in U.S. Patents No. 5,270,184, No. 5,455,166 and No. 5,648,211, which are incorporated by reference herein in their entirety. A SDA assay can be, but is not limited to, a traditional (or conventional) SDA (as described in Walker *et al.*, PNAS (1992) 89:392-396, U.S. Patent Nos. 5,962,273, 5,712,124, and 5,744,311, each of which is incorporated herein by reference), a thermophilic SDA (as described in Walker *et al.*, Nuc. Acids Res. (1992) 20:1691-1696, U.S. Patent Nos. 5,648,211 and 5,744,311, each of which is incorporated herein by reference), and a homogeneous real time fluorescent thermophilic SDA (as described in U.S. Patent No. 6,379,888, which is incorporated herein by reference).

[027] Cross-contamination with amplification products carried over from previous amplification reactions in reagents, pipetting devices and laboratory surfaces may be reduced by incorporating various residues into extension products. For example, thymine may be substituted with 2'-deoxyuridine 5' triphosphate ("dU"), as is taught in EP 0 624 643. Excision of dU that is incorporated into amplification products is catalyzed by uracil DNA glycosylase ("UDG"), which renders amplification products containing dU incapable of further amplification. The UDG itself may be inactivated when appropriate to continue amplification.

[028] In the case of tSDA, primers and their target sequences preferably are selected such that their GC content is less than 70% of the total nucleotide composition to minimize secondary structure and primer-primer interactions that may limit target amplification efficiency. A suitable amplification primer for tSDA comprises, in order from the 3' end of the probe to the 5' end, a target binding sequence, a restriction endonuclease recognition site, and a "tail." The target binding sequence hybridizes specifically to a complementary sequence of the target nucleic acid. The restriction endonuclease recognition site is recognized by a restriction endonuclease that nicks one strand of a DNA duplex when the

recognition site is hemimodified, as described by Walker *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:392 (1992) and Walker *et al.*, *Nucl. Acids. Res.* 20:1691 (1992). The 5' tail functions as a polymerase repriming site when the remainder of the amplification primer is nicked and displaced during tSDA. The repriming function of the tail sustains the tSDA reaction and allows synthesis of multiple amplicons from a single target molecule. The length and sequence of the tail region may vary, provided that the tail remains hybridized to the target after nicking and that the tail does not contain sequences that will hybridize either to the target binding sequence or to other primers.

[029] Some amplification methods, such as tSDA, use a "bumper primer" or "external primer" to displace primer extension products. A "bumper primer" or "external primer" is a primer used to displace an amplification primer and its extension product in an amplification reaction. A bumper primer anneals to a target sequence upstream of an amplification primer such that extension of the bumper primer displaces the downstream amplification primer and its extension product. Primer extension products alternatively may be displaced by heating. Bumper primers used in SDA and tSDA reactions need not hybridize specifically to enterovirus nucleic acids. Rather, bumper primers may hybridize to any target sequence that is upstream from the amplification primers and that is sufficiently close to the binding site of the amplification primer to displace the amplification primer extension product upon extension of the bumper primer. Mismatches between the bumper primer sequence and its target sequence generally do not affect amplification efficiency, provided the bumper primer still hybridizes to the its target sequence. Furthermore, the specificity of the SDA system for amplification of the target sequence in preference to other nucleic acids is not dependent upon the specificity of the bumper primer(s) for hybridization to the target nucleic acid. The specificity of an SDA system for the target sequence is derived from the fidelity of hybridization of the SDA primers and probes or oligonucleotides used for detection of amplified products.

[030] When an amplification reaction used in accordance with the invention is a tSDA reaction, the polymerases that can be used include, but are not limited to, exo$^-$ Vent (New England Biolabs), exo$^-$ Deep Vent (New England Biolabs), Bst (BioRad), exo$^-$ Pfu (Stratagene), Bca (Panvera), and Sequencing Grade Taq (Promega). Others may be routinely identified using the foregoing extension assay. The polymerases Tth (Boehringer), Tfi (Epicentre), REPLINASE (DuPont) and REPLITHERM (Epicentre) are able to displace a strand from a nick, but they also have 5'-3' exonuclease activity. These polymerases are useful in the methods of the invention after removal of the exonuclease activity, *e.g.*, by

genetic engineering. As the thermostability of thermophilic restriction endonucleases is generally limited to less than 65°C, thermophilic polymerases with optimal activity around this temperature or lower (*e.g.*, Bst and Bca) are more compatible with thermophilic restriction endonucleases in the reaction.

[031] The components of the present invention may be optimized to a form where each component could be dried and rehydrated when needed by using any technique known in the art. (*See* Little *et al.*, Clinical Chemistry 45(6):777-784 (1999), which is incorporated herein by reference).

Primer Design

[032] An "amplification primer" is an oligonucleotide for amplification of a target sequence by extension of the oligonucleotide after hybridization to a target sequence or by ligation of multiple oligonucleotides that are adjacent when hybridized to the target sequence. At least a portion of the amplification primer hybridizes to the target sequence. This portion is referred to as the target binding sequence and it determines target-specificity of the primer. It should be understood that the target binding sequences exemplified in the present invention may also be used in a variety of other ways for detection of *Neisseria gonorrhoea.* For example, the target binding sequences disclosed herein may alternatively be used as hybridization probes for direct detection of *Neisseria gonorrhoeae*, either without prior amplification or in a post amplification assay. Such hybridization methods are well known in the art and typically employ a detectable label associated with or linked to the target binding sequence to facilitate detection of hybridization.

[033] The design of amplification primers may be optimized for each method of amplification. As no special sequences or structures are required to drive the amplification reaction, amplification primers for a Polymerase Chain Reaction (PCR) may consist only of template binding sequences. However, other amplification reactions require specialized nucleotide sequences, in addition to the target binding sequence, in order for the reaction to proceed. For example, an amplification primer for use in a SDA assay further comprises a restriction endonuclease recognition site 5' to the target binding sequence (see U.S. Patent Nos 5,455,166 and 5,270,184). The amplification primer may also comprise a 3'-OH group, which is extendable by DNA polymerase when the template-binding sequence of the amplification primer is annealed to the target sequence. Amplification primers for Self-sustained Sequence Replication (3SR) and Nucleic Acid Sequence-Based Assay (NASBA), in contrast, comprise an RNA polymerase promoter near the 5' end. (3SR assays are

described in Guatelli *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878) The promoter is appended to the target binding sequence and serves to drive the amplification reaction by directing transcription of multiple RNA copies of the template. Such sequences in additional to the target binding sequence that are necessary for a particular amplification reaction are well known in the art. a promoter recognized by RNA polymerase for self-sustained replication assays

[034] In designing the amplification primers and the bumper primers of the present invention, general concerns known in the art should be taken into account. For example, when a target sequence comprising a large number of GC and AT repeats is used for designing a primer, cares should be taken to minimize potential dimer interactions to avoid self-hybridization of primers. Primers that can form four or more consecutive bonds with itself, or eight or more inter-strand bonds with other primers should be generally avoided. Primers that can form 3' dimers should especially be avoided, because hybridizing at the 3' ends of the primer, even transiently, will lead to extension of the primer due to polymerase action and ruining of the primer. Certain computer software programs (*e.g.*, Oligo™, National Biosciences, Inc., Plymouth, Minn) can be used in designing of the primers to avoid the problems. Primer combinations are also screened for optimal conditions.

[035] As is known in the art, annealing or hybridization of complementary and partially complementary nucleic acid sequences may also be obtained by adjustment of the reaction conditions to increase or decrease stringency (*e.g.*, adjustment of temperature or salt content of the buffer). Such modifications of the disclosed sequences and any necessary adjustments of conditions are encompassed by the present invention. Information relating to buffer conditions can be found in Experimental Design in Biotechnology by Dr. Perry Haaland (Marcell Dekker, NY, 1989), incorporated herein by reference in its entirety.

[036] In a diplex amplification reaction, an amplification primer is designed to be able to hybridize to both a *Neisseria gonorrhoeae* target sequence and an IAC sequence and amplify the sequence to which it is hybridized. This is achieved by using a shared nucleic acid sequence between *Neisseria gonorrhoeae* target sequence and an IAC sequence to design an amplification primer. Other sequences, as required for performance of a selected amplification reaction, may optionally be added to an amplification primer as disclosed herein.

[037] By way of example, but not limitation, amplification primers for use in a SDA assay generally comprise a 3' template-binding sequence, a nickable restriction endonuclease

recognition site 5' to the template-binding sequence, and a tail sequence about 10-25 nucleotides in length 5' to the restriction endonuclease recognition site. Such amplification primer may contain a recognition site for the restriction endonuclease *Bso*BI, which is nicked during the SDA reaction. It will be apparent to one skilled in the art that other nickable restriction endonuclease recognition sites may be substituted for the *Bso*BI recognition site. The amplification primer may also contain a tail sequence (5' to the restriction endonuclease recognition site). The tail sequence should not contain the restriction site used for SDA and sequences which will anneal either to its own target binding sequence or to the other primers (*e.g.*, bumper primers).

[038] In some embodiments, a pair of amplification primers is used, each of which anneals to one of the two strands of a double stranded target sequence or IAC sequence. In this case, amplification is exponential because both the sense and antisense strands serve as templates for the opposite primer in subsequent rounds of amplification. When a single amplification primer is used, amplification is linear because only one strand serves as a template for primer extension.

[039] In some embodiments, the methods of the present invention encompass an amplification primer that comprises a nucleotide sequence consisting essentially of SEQ ID NO: 1, 2, 10, 11, 12 , 13 or their respective target binding sequences. In other embodiments, the methods of the present invention encompass at least two amplification primers, wherein a first amplification primer comprises a nucleotide sequence consisting essentially of SEQ ID NO: 1, 10, 11 or their respective target binding sequences; and a second amplification primer comprises a nucleotide sequence consisting essentially of SEQ ID NO: 2, 12, 13 or their respective target binding sequences.

[040] In some embodiments, the methods of the present invention encompasses one or more bumper primers. A bumper primer is a primer used to displace an amplification primer and its extension product in an amplification reaction. A bumper primer anneals to a target sequence upstream of an amplification primer, such that extension of the bumper primer displaces the downstream amplification primer and its extension product. A bumper primer may also function as an amplification primer. In some embodiments, the methods of the present invention encompass one or more bumper primers. In certain embodiments, the bumper primer comprises an oligonucleotide having the sequence comprising SEQ ID NO: 3, 4, 14, 15, 16 or 17. In one embodiment, a bumper primer comprises an oligonucleotide having a partial or complete sequence of SEQ ID NO: 3, 4, 14, 15, 16 or 17. In another embodiment, the methods of the present invention encompass at least two bumper primers,

wherein a first primer comprises a nucleotide sequence consisting essentially of SEQ ID NO: 3, 14, or 15, and a second primer comprises a nucleotide sequence consisting essentially of SEQ ID NO: 4, 16, or 17.

Target Sequences

[041] "Target" or "target sequence" refers to a *Neisseria gonorrhoeae* nucleic acid sequence to be amplified and/or detected. A target or target sequence includes the *Neisseria gonorrhoeae* nucleic acid sequence to be amplified and any complementary second strand. In some embodiments, a target sequence may be single-stranded or double-stranded, in which case, either one or both strands can bind to an amplification primer. A target or target sequence may also comprise a nucleotide sequence that is recognized by an adapter oligonucleotide (*i.e.*, adapter-binding sequence).

[042] The primers of the present invention are designed to anneal to a region of *Neisseria gonorrhoeae* genomic DNA illustrated in Figure 1. (see U.S. Patent No. 5,962,273, which is incorporated herein by reference in its entirety).

Internal Amplification Control

[043] "Internal amplification control", "IAC" or "IAC sequence" refers to a nucleic acid sequence comprising a sequence that anneals to an amplification primer and a sequence that can be detected separately from the target sequence. Any detection method known in the art may be employed.

[044] In accordance with the present invention, an IAC sequence is designed to share nucleic acid sequences with a *Neisseria gonorrhoeae* target sequence, thus the same amplification primer(s) can amplify both an IAC sequence and a target sequence if it is present in a sample. An IAC sequence is also designed to have some nucleic acid sequences that differ from a *Neisseria gonorrhoeae* target sequence, so that the detection of the IAC sequence and the target sequence may be differentiated. Since an IAC sequence is amplified and/or detected in the same reaction mixture as a target sequence, diplex assays have the advantage of detecting human error or an inhibitory reaction condition, *e.g.*, the presence of an inhibitor or absence of a critical reagent. The presence of an IAC in the same reaction as the sample to be tested eliminates the need for separate amplification control reactions as required by the current monoplex SDA assays.

[045] Although not intending to be bound by a particular mechanism of action, the presence of an IAC in the same reaction as a target sequence allows the amplification assay

of the present invention to detect the presence of inhibitors of the reaction and/or conditions that may indicate a false negative result. As used herein, a false negative result refers to a result that indicates no detection of a target sequence, however, such indication is not due to the absence of the target sequence in the sample, but due to human error or a reaction condition, *e.g.*, the lack of a critical reaction element, or the existence of an inhibitor of the reaction, or a mistake in performing the assay.

[046] A detection method is used wherein such method differentiates amplification products of a target sequence from amplification products of an IAC sequence. In one embodiment, the amplification products of the target sequence and the IAC may be detected by different dye labeled detection probes. In another embodiment, fluorescein is used to detect amplification products of the target sequence and rhodamine fluorescence is used to detect the amplification products of the IAC.

[047] In some embodiments, an IAC sequence is designed such that either its 3' or 5' terminus contains a sequence in common with a *Neisseria gonorrhoeae* DNA sequence. In some other embodiments, an IAC is designed such that both the 3′ and 5′ terminus contain sequences in common with a *Neisseria gonorrhoeae* DNA sequence for an amplification primer to bind.

[048] An IAC sequence is also designed to comprise a nucleic acid sequence that is different from the *Neisseria gonorrhoeae* target sequence to be amplified, such that the detection of the amplification products of the IAC and the target sequence can be differentiated.

[049] In some embodiments, the methods of the present invention utilize an IAC that comprises a nucleotide sequence consisting essentially of SEQ ID NO: 9 or 19.

<u>Detection of Nucleic Acids</u>

[050] The amplification products generated using one or more primers of the invention can be detected by any method known in the art. As used herein, amplification products include both the amplified target sequences and the amplified IAC sequences. Amplification products can be detected by hybridization to a labeled probe using conventional techniques, for example, one that hybridizes to amplified nucleic acids at a sequence that lies between the amplification primers. Alternatively, amplification products may be detected by their characteristic size, for example by electrophoresis followed by ethidium bromide staining to visualize the nucleic acids. In a further alternative, a labeled

amplification primer is used. In a still further alternative, a labeled amplification primer/internal probe is extended on the target sequence, as described by Walker *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:392 (1992); or Walker *et al.*, *Nucl. Acids Res.* 20:1691 (1992). In another embodiment, detection is accomplished directly through hybridization and extension of a labeled reporter probe as described in US Patent No. 5,928,869 and US Patent No. 5,958,700. Detection methods also include a chemiluminescent method in which amplified products are detected using a biotinylated capture probe and an enzyme-conjugated detector probe, as described in U.S. Patent No. 5,470,723. After hybridization of these two probes at different sites between the two amplification primer binding sites, the complex is captured on a streptavidin-coated microtiter plate, and the chemiluminescent signal is developed and read in a luminometer.

[051] In an embodiment of the present invention, the detection method should detect both the target and the IAC amplification products, and differentiate between the amplification products detected. Any method known in the art capable of achieving this purpose can be used. For example, the detection methods that are disclosed in Walker *et al.*, Nucl. Acids Res., (1992) 20:1691-1696, the U.S. Patent Nos. 5,648,211, 5,962,273, 5,814,490, 5,928,869, 6,316,200, and European Patent EP 0 678 582 (each of which is incorporated herein by reference) can be used in accordance with the present invention. In another embodiment, universal probes and methods for detection of nucleic acids are used (*see* U.S. Patent No. 6,379,888, which is incorporated herein by reference).

[052] In one embodiment, the universal detection method employs an adapter oligonucleotide and a detection probe for detection of a target sequence (a target sequence, an IAC sequence, or extension products thereof). The adapter oligonucleotide comprises a 3' target binding sequence and a 5' sequence. The complement of the 5' sequence, produced by amplification, will hybridize and extend off of the detection probe. Such hybridization can than be detected as an indication of a successful amplification reaction.

[053] At least two different adapter oligonucleotides may be used to simultaneously detect different target sequences (*e.g.*, amplification products of a target sequence and amplification products of an IAC sequence). In this case, the 5' adapter sequence of the adapter oligonucleotide is different for each template to be detected. By labeling detection probes which indicate the presence of target sequence and IAC with different fluorescent labels (*e.g.*, different donor/quencher dye pairs), the presence of each can be determined by detecting changes in the extent of fluorescence quenching in each of the detection probes.

[054] In some embodiments, adapter oligonucleotides and detection probes are used for the detection of the amplification products of an IAC and a target sequence. The portion of the IAC sequence that can be recognized by an adapter oligonucleotide is designed to be different from the portion of the target sequence that can be recognized by an adapter oligonucleotide, *i.e.*, the adapter oligonucleotide that recognizes the IAC sequence and the adapter oligonucleotide that recognizes the target sequence have, *inter alia*, different template-binding sequences. The specificity of the adapter oligonucleotides (with respect to the IAC sequence and the target sequence) ultimately leads to the detection of different amplification products (of an IAC or of a target sequence) by different detection probes.

[055] In one embodiment, multiple adapter oligonucleotides per strand of template may be employed if desired, each hybridizing to the target sequence downstream of the other on the same strand, with all adapter oligonucleotides being hybridized downstream of the amplification primer. In this manner, each adapter oligonucleotide is displaced by extension of the upstream adapter oligonucleotide and the most 5' adapter oligonucleotide is displaced by the amplification primer. Use of multiple adapter oligonucleotides has the advantage of increasing or amplifying the signal generated per target sequence, with an increase in sensitivity of the assay.

[056] Many donor/quencher dye pairs known in the art are useful in the present invention. These include, but not limited to, for example, fluorescein isothiocyanate (FITC)/tetramethylrhodamine isothiocyanate (TRITC), FITC/Texas Red.TM. (Molecular Probes), FITC/N-hydroxysuccinimidyl 1-pyrenebutyrate (PYB), FITC/eosin isothiocyanate (EITC), N-Docket hydroxysuccinimidyl 1-pyrenesulfonate (PYS)/FITC, FITC/Rhodamine X, FITC/tetramethylrhodamine (TAMRA), and others. The selection of a particular donor/quencher pair is not critical. For energy transfer quenching mechanisms it is only necessary that the emission wavelengths of the donor fluorophore overlap the excitation wavelengths of the quencher, *i.e.*, there must be sufficient spectral overlap between the two dyes to allow efficient energy transfer, charge transfer or fluorescence quenching. P-(dimethyl aminophenylazo) benzoic acid (DABCYL) is a non-fluorescent quencher dye which effectively quenches fluorescence from an adjacent fluorophore, *e.g.*, fluorescein or 5-(2'-aminoethyl) aminonaphthalene (EDANS). Any dye pair which produces fluorescence quenching in the detection probe of the invention can be used in the methods of the invention, regardless of the mechanism by which quenching occurs. Terminal and internal-labeling methods are also known in the art and may be routinely used to link the donor and quencher dyes at their respective sites in the detection probe.

[057] In some embodiments, the methods of the present invention utilize an adapter oligonucleotide that comprises a nucleotide sequence consisting essentially of SEQ ID NO: 5, 6, 18, 20 or 21.

[058] The present invention provides detection probes that are single-stranded oligonucleotides comprising SEQ ID NO: 7, 8, 22 or 23, and a label. In certain embodiments, the label comprises at least one fluorescent donor/quencher pair linked to the oligonucleotide, wherein the fluorescent moiety is rhodamine, fluorescein or dabcyl.

[059] In some embodiments, the present invention provides diplex homogeneous real time fluorescent thermophilic SDA (tSDA). Homogeneous real time fluorescent thermophilic SDA is a modified tSDA which detects nucleic acid target sequences by fluorescence quenching mechanisms (*see*, *e.g.*, U.S. Patent No. 6,379,888, which is incorporated herein by reference in its entirety). For example, in one embodiment, a detection probe may comprise a fluorescent donor/acceptor pair so that fluorescence quenching occurs in the absence of a target sequence. Although not intending to be bound by a particular mechanism of action, in the absence of hybridization of the detection probe to a second oligonucleotide (which is produced by amplification of a target sequence), the probe adopts a conformation which brings the donor and quencher into close spatial proximity and results in quenching of donor fluorescence. The probe may fold into an ordered secondary structure (*e.g.*, a G-quartet, hairpin or triple helix), into a random coil, or into any other conformation which brings the donor and quencher into close enough proximity to produce fluorescence quenching. However, when the detection probe hybridizes to a second oligonucleotide, the intramolecularly base-paired secondary structure of the detection probe becomes unfolded or linearized, which increases the distance between the donor and the quencher and thereby reducing or eliminating fluorescence quenching. Alternatively, the detection probe may be designed as a linear detection probe (*i.e.*, it does not fold into a secondary structure), wherein the distance between the donor and the quencher is short enough to produce fluorescence quenching. In this case (and optionally in cases where a non-linear detection probe described herein is used), the detection probe also contains a restriction endonuclease recognition site (RERS) between the fluorescent donor/quencher pair. The intermolecular base-pairing between the detection probe and a second oligonucleotide renders the RERS double-stranded and thereby cleavable or nickable by a restriction endonuclease. Although not intending to be bound by a particular mechanism of action, cleavage or nicking by the restriction endonuclease separates the donor and acceptor onto separate nucleic acid fragments, which leads to decreased quenching.

[060] An associated change in a fluorescence parameter (*e.g.*, an increase in donor fluorescence intensity, a decrease in acceptor fluorescence intensity or a ratio of the donor and/or acceptor fluorescence intensities) may be monitored in accordance with the methods of the invention to detect and/or monitor the presence of the target sequence. Monitoring a change in donor fluorescence intensity is usually preferred, as this change is typically larger than the change in acceptor fluorescence intensity. Other fluorescence parameters such as a change in fluorescence lifetime may also be monitored in accordance with the invention.

Kits

[061] The present invention also provides kits for amplification and/or detection of *Neisseria gonorrhoeae* nucleic acids comprising one or more amplification primers consisting essentially of SEQ ID NOS:1, 2, 10, 11, 12, 13 or their respective target binding sequences and at least one container which contains such primers. The kit may optionally include any one or more of: an IAC, adapter oligonucleotides, or detection probes. The kit may further include other components and reagents for performing a hybridization or amplification reaction, such as a Southern hybridization, dot blot hybridization, PCR, or SDA. For detection by hybridization, a appropriate solution to perform hybridization may be included, *e.g.,* 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS. Components for detection methods also may be included in the kit, *e.g.,* a second probe, a radiolabel, an enzyme substrate, an antibody and the like. Reagents appropriate for use with a nucleic acid amplification method also may be included. The components of the kit are packaged together in a common container, typically including instructions for performing selected specific embodiments of the methods disclosed herein.

EXAMPLES

Example 1: Design of SDA Primer Sets

[062] A portion of the *Neisseria gonorrhoeae* genome has been sequenced and characterized for targeting by amplification assays (*see* U.S. Patent No. 5,962,273, incorporated herein by reference in its entirety). For purpose of this assay, a portion of the *Neisseria gonorrhoeae* genome that had not previously been targeted for amplification assays was selected for targeting. This sub-region of the *Neisseria gonorrhoeae* genome was analyzed in current GenBank and SeqWeb databases for *Neisseria gonorrhoeae* specificity.

[063] Amplification primers were designed to amplify both *Neisseria gonorrhoeae* target sequences and an IAC. Multiple versions of the primers were designed as shown in Table 2 and 3. The positions of the regions of the *Neisseria gonorrhoeae* genome to which the selected oligonucleotides (amplification primers, bumper primers, and adapter oligonucleotides) anneal are illustrated in Figure 1.

Table 2. Primary Oligonucleotides of SDA Assays for Amplification and/or Detection of *Neisseria gonorrhoeae*

Oligonucleotide	**Sequence**
Amplification Primers:	
GCINT3-APL2	5'CGTCTCCAGTCCAGACTT*CTCGGG*AATCAAAAGCGAATGCG3' (SEQ ID NO: 1)
GCINT3-APR1	5'ACTACGTCGAATGCATGT*CTCGGG*ACTTCTTCATCTTTTGC3' (SEQ ID NO:2)
Bumpers:	
GCINT3-BL2	5'CCGCAGCATACGC3' (SEQ ID NO:3)
GCINT3-BR3	5'TGCGCATATGCTTTG3' (SEQ ID NO:4)
Adapter Oligonucleotides:	
GC3A-R1	5'**ACGTTAGCCACCATACTTGA**GTGATGACGGTTTTTCATTGC3' (SEQ ID NO:5)
ICAdpt9	5'**ACTGATCCGCACTAACGACTG**CTTTGCTAGTTGCCTCAGACAT3' (SEQ ID No. 6)
GC3A-R2	5' **ACTGATCCGCACTAACGACT**GTGATGACGGTTTTTCATTGC 3' (SEQ ID NO:20)
ICAdpt10	5' **ACGTTAGCCACCATACTTGA**GCTTTGCTAGTTGCCTCAGACAT 3' (SEQ ID NO:21)
Detection probes:	
CTGCUnv3	5' (Dabcyl)- TAGTGC*CCGAGC*AC (Rhodamine)-T**ACGTTAGCCACCATACTTGA**3' (SEQ ID NO:7)
ICUnv4	5' (Fluorescein)- TAGCA*CCCGAG*TGC (Dabcyl)-T**AACTGATCCGCACTAACGACT**3' (SEQ ID NO:8)
MPC-DR	5' (Dabcyl)- TC*CCCGAG*T-(Rhodamine)**ACGTTAGCCACCATACTTGA** 3' (SEQ ID NO:22)
MPC2-FD	5' (Fluorescein)- TC*CCCGAG* T-(Dabcyl) **ACTGATCCGCACTAACGACT** 3' (SEQ ID NO:23)
IAC:	
GCI3-IAC4	5'AATCAAAAGCGAATGCGTATGTCTGAGGCAACTAGCAAAGCT

	GCAAAAGATGAAGAAG3' (SEQ ID NO:9)

Universal detection sequences are in bold and *BsoB*1 sites in italic.
Underlined represents target binding sequences.

Table 3. Additional Oligonucleotides of SDA Assays for Amplification and/or Detection of *Neisseria gonorrhoeae*

Oligonucleotide	Sequence
Amplification Primers:	
GCINT3-APL1	5'CGTCTCCAGTCCAGACTT*CTCGGG*AATCAAAAGCGAATGC3' (SEQ ID NO: 10)
GCINT3-APL3	5'CGTCTACCGTCCAGACTT*CTCGGG*AATCAAAAGCGAATGCGC3' (SEQ ID NO:11)
GCINT3-APR2	5'ACTACGTCGAATGCATGT*CTCGGG*ACTTCTTCATCTTTTGCC3' (SEQ ID NO:12)
GCINT3-APR3	5'ACTACGTCGAATGCATGT*CTCGGG*AGCTTCTTCATCTTTTGCC3' (SEQ ID NO:13)
Bumpers:	
GCINT3-BL1	5'CCGCAGCATACG3' (SEQ ID NO:14)
GCINT3-BL3	5'CCGCAGCATACGCG3' (SEQ ID NO:15)
GCINT3-BR1	5'TTGCGCATATGCTT3' (SEQ ID NO:16)
GCINT3-BR2	5'CTTTGATGATTTGCG3' (SEQ ID NO:17)
Adapter Oligonucleotides:	
GC3A-F1	5'**ACGTTAGCCACCATACTTGA**GCAATGAAAAACCGTCATCAC3' (SEQ ID NO: 18)
IAC	
IC3-IAC3	5' AATCAAAAGCGAATGCGTATGTCTGAGGCAACTAGCAAA GCAGTGCAAAAGATGAAGAAG 3' (SEQ ID NO:19)

Universal detection sequences are in bold and *BsoB*1 sites are in italic.
Underlined represents target binding sequences.

Example 2: Optimization of SDA Reaction Conditions

[064] Homogeneous real time fluorescent tSDA assays were performed to optimize the conditions for the assays of the invention. The oligonucleotides described in Table 2 and 3 were assayed to determine which oligonucleotides would provide optimal results. Concentrations of bicine and potassium hydroxide were also optimized. An example of optimized concentrations of the components of the SDA assay is as follows:

50nM bumper (GCINT3-BL2 and BR3)
500nM amplification primer (GCINT3-APL2 and APR1)
200nM adapter (GC3A-R1 and ICADPT9)
200nM detection probes (CTGCUnv3 and ICUnv4)

30mM potassium phosphate
99.7mM bicine
58mM potassium hydroxide
10% DMSO
9% glycerol
0.1mM dATP, 0.1mM dGTP, 0.25mM dUTP and.0.35mM S isomer of alpha thioated dCTP
5mM magnesium acetate
100ug/ml BSA
1.82% trehalose
360uM DTT
15 BD units BsoB 1
6 BD units Bst

[065] Target DNA was lysed for 10 minutes at 114°C in a BD Lyse Block. Samples were cooled for 10 minutes and 150 μl was transferred to priming wells to incubate for 20 minutes. 100μl of the SDA priming mix was transferred to amplification microwells. The plates were then transferred into the BDProbeTec™- ET instrument for 60 minutes at 54°C.

[066] Some oligonucleotides listed in Table 2 and Table 3 (*e.g.* APL 1 and APL2) were equivalent in performance, *i.e.*, when these oligonucleotides were used in a SDA assay, the amount of amplification products detected are about the same (or the variation is within a statistical significance). However, GC3A-R1 provided a stronger detection signal as compared to GC3A-F1 when the same amount of target sequence was present. Amplification primers include GCINT3-APL2 (SEQ ID NO:1) and GCINT3-APR1 (SEQ ID NO:2). Bumper primers include GCINT3-BL2 (SEQ ID NO:3) and GCINT3-BR3 (SEQ ID NO:4). Adapter oligonucleotide include GC3A-R1 (SEQ ID NO:5), IACAdpt9 (SEQ ID NO:6), GC3A-R2 (SEQ ID NO:20), and ICAdpt10 (SEQ ID NO:21). Detection probes include

CTGCUnv3 (SEQ ID NO:7), ICUnv4 (SEQ ID NO:8), MPC-DR (SEQ ID NO:22), and MPC2-FD (SEQ ID NO:23). IAC includes GCI3-IAC4 (SEQ ID NO:9).

<u>SDA Assays with Dry Components and Linear Detection Probes</u>

[067] The components of the SDA assays in accordance with the present invention were optimized to a format where each component could be dried and rehydrated when needed. The concentrations of oligonucleotides and reagents for a 100μl SDA reaction are listed below.

<u>Oligonucleotides utilized and their concentrations</u>:
50nM of bumper GCINT3-BL2
50nM of bumper GCINT3-BR3
500nM of amplification primer GCINT3-APL2
200nM of amplification primer GCINT3-APR1
130nM of adapter GC3A-R2
200nM of adapter ICAdpt10
300nM of detector MPC-FD
300nM of detector MPC2-DR
300 copies of GCI3-IAC4

<u>Concentrations of reagent and other components</u>:
60mM potassium phosphate, (made from pH7.6 stock)
10% DMSO
9% glycerol
0.1mM dATP, 0.1mM dGTP, 0.1mM dTTP, and 0.125mM S isomer of α thioated dCTP
3.5mM magnesium acetate
100ug/ml BSA
1.84% trehalose
320mM DTT
48 BD units BsoB1 restriction enzyme
8.4 BD units Bst polymerase

[068] This optimized reaction mix utilized GC3A-R2 (SEQ ID NO:20) and ICAdpt10 (SEQ ID NO:21) as adapter oligonucleotides, and MPC-DR (SEQ ID NO:22) and MPC2-FD (SEQ ID NO:23) as detection probes. The *Neisseria gonorrhoeae* target DNA amplification products were detected by MPC2-FD, which was labeled with fluorescein and dabcyl. The IAC (GCI3-IAC4) DNA amplification products were detected by MPC-DR, which was labeled with rhodamine and dabcyl. The detection probes MPC-DR and MPC2-FD do not need intramolecular hybridization (*e.g.*, folding into a hairpin structure) to achieve quenching of the fluorescent dyes, since the dye and the quencher molecule are positioned in

close proximity. A *BsoB*1 restriction site is present between the dye and the quencher molecule. Amplification of the *Neisseria gonorrhoeae* target DNA or the IAC leads to the generation of a second oligonucleotide complementary to the adapter oligonucleotide. The second oligonucleotide, which is complementary to the detection probe, anneals to the detection probe and generates a double strand DNA. The *BsoB*1 restriction enzyme recognizes the double stranded *BsoB*1 site between the dye and the quencher molecule and cleaves the site, which separates the fluorescent dye from the quenching molecule and generates the signal to be detected.

Example 3: Assay Sensitivity

[069] The optimized reaction conditions described in Example 2 (see paragraph 064) were used to determine the sensitivity of a diplex SDA assay in accordance with the present invention. A strain of *Neisseria gonorrhoeae* (ATCC# 19424) was titrated from 1×10^4 to 25 particles per SDA reaction. The *Neisseria gonorrhoeae* cells were lysed in a BD lyse block, added to the SDA reaction mixture, and then amplified in the BDProbeTec™ ET instrument. One hundred percent of the diplex SDA reactions containing *Neisseria gonorrhoeae* were positive, with detection values significantly greater than those seen for the negative control, and thus confirming that the diplex SDA assay of the invention is sensitive for *Neisseria gonorrhoeae* nucleic acid.

Table 4. *Neisseria gonorrhoeae* Limit of Detection Experiment

Cells/reaction:	10,000	1,000	500	100	50	25	0
MOTA:	76,990	97,390	92,160	86,960	82,290	78,980	450
	91,020	79,160	86,800	94,700	80,190	77,990	300
	17,910	98,710	99,150	86,920	94,000	69,940	710
	88,880	101,920	94,960	94,190	86,820	71,050	60
	10,1790	103,940	98,000	92,490	94,040	66,150	810
	85,750	100,460	88,840	92,880	94,730	72,240	40
	106,660	100,550	84,360	85,880	86,200	90,580	360
	99,990	99,990	91,600	90,360	78,380	79,340	140
Mean:	**96124**	**97754**	**91984**	**90548**	**87956**	**75784**	**359**

[070] The optimized dry/rehydration reaction condition described in Example 2 (see paragraph 067) was also tested for its sensitivity. Different samples, which contained

Neisseria gonorrhoeae cells (strain 19424) with different concentrations in 2 mls of buffer [30mM potassium phosphate buffer (made from pH7.6 stock), 10% DMSO, and 9% glycerol], were lysed for 10 minutes at 114°C in a BD Lyse Block. Samples were cooled for 15 minutes and 150μl of each sample was transferred to the priming wells. The rehydrated samples in the priming wells were incubated for 20 minutes at room temperature followed by 10 minutes on a heat block set at 72.5°C. For each sample, 100μl of the priming reaction mix was transferred to an amplification microwell previously heated for 10 minutes on a 54°C heat block, followed by mixing 50μl of the transferred volume three times in the amplification well. The plates were sealed and transferred into the BDProbeTec™ ET instrument for 60 minutes at 52°C. Specimens having a reading of at least 4,000 MOTA (as determined by a Method Other Than Acceleration) were recorded as positive for *Neisseria gonorrhoeae* (*i.e.*, the 4,000 MOTA reading was the cut-off value for a sample to be determined positive for *Neisseria gonorrhoeae*). MOTA is described in published European application no. 01110657.2, which is hereby incorporated by reference. The highest value that a sample negative for *Neisseria gonorrhoeae* target DNA achieved was 780 MOTA. The MOTA readings were obtained according to the BDProbeTec™ ET (Becton Dickinson, MD) manufacturer's instructions.

[071] The data in Table 5 indicates that the diplex SDA assay according to the present invention can detect at least 5 *Neisseria gonorrhoeae* particles per reaction, in which the 95% LOD would be 8 copies per reaction.

Table 5. Analytical Sensitivity of Diplex SDA Assay for *Neisseria gonorrhoeae* with Optimized Drydown Reaction Mix

GC particles/ reaction:	100		50		25		10		5		0	
Assay:	**GC**	**IAC**	**GC**	**IAC**	**GC**	**IAC**	**GC**	**IAC**	**GC**	**IAC**	**GC**	**IAC**
MOTA Signal:	35310	53810	29590	13020	29030	62760	17430	33260	1450	51720	70	23970
	40430	43470	37330	22980	29370	60730	21520	45650	7440	67860	50	28710
	44710	47850	30740	34010	38490	62380	21350	52550	30400	44520	0	27810
	42830	48900	41850	40070	26130	68260	20370	59350	16780	61070	0	33420
	45890	56330	33720	21230	37780	64060	24750	61590	10200	62770	0	31800
	46440	45940	32840	37910	11310	63130	9690	61080	9400	55130	0	21200
	41760	52050	27620	39700	44480	52060	6680	61080	9870	46970	0	24610
	45030	40540	28410	38390	26790	57070	4860	63420	37170	40580	40	25490
	47150	57830	25470	21300	27220	46500	20550	36150	21810	74400	10	30800

	52600	54100	25350	15400	28520	60980	13530	38000	8990	75350	0	36760
	46430	42930	26160	31500	13390	67020	4400	46070	11260	68370	20	33630
	37050	52130	20190	34580	13160	69200	22570	46970	800	69120	0	29870
	34160	52800	23670	25080	24550	46050	20930	30400	4250	69090	50	34660
	20010	27110	33000	17740	21280	61770	13980	44680	3780	57160	400	23850
	25030	25730	26260	37050	14160	54520	14990	43910	5640	48400	500	27940
	27650	43420	14870	43620	19400	49540	25170	37440	9280	39860	0	27460
	34430	16280	20170	11350	9410	60260	4980	37570	2190	50000	10	31130
	26700	23730	16510	23140	19250	56410	10520	44460	9130	52690	0	40930
	31450	29120	22860	15220	17710	56680	5650	46490	25800	40170	200	39490
	37310	35120	20210	27470	21120	53100	12180	51870	8220	50060	0	36480
	30480	40810	20400	17350	13670	60860	6640	50260	6070	43700	10	41690
			28530	18160	17810	44850	26520	42950	2050	59640	290	39220
			14210	31980	16390	47370	20220	41150	20010	14720	780	45520
			21600	18020	16010	35580	24280	33250	12640	43040		
Average:	37,755	42,381	25,898	26,511	22,351	56,714	15,573	46,233	11,443	53,600	106	32,019

<u>Example 4: Assay of *Neisseria gonorrhoeae* and Non *Neisseria gonorrhoeae* Species Specificity</u>

[072] The specificity of the diplex SDA assay of the invention was determined using the oligonucleotides in Table 2 and the SDA reaction conditions described in Example 2: SDA Assays with Dry Components and Linear Detection Probes.

[073] A panel of *Neisseria gonorrhoeae* strains was tested to determine the specificity of the diplex SDA assay of the invention. The assay detected one hundred percent of the *N. gonorrhoeae* strains when 250 cell particles per SDA reaction was tested (Table 6). A panel of *Neisseria* but non *gonorrhoeae* species and non *Neisseria* microorganisms were also tested in the diplex SDA assay of the invention (Table 7 and Table 8). The results in the tables are a summary of multiple experiments that addressed crossreactivity. The organisms were grown to one McFarland which, after processing, yielded approximately 1×10^7 cells/ SDA reaction. The organisms listed in Table 7 and 8 were tested at that concentration, with the exception being *C. trachomatis* at 1×10^6 EBs/ reaction. The IAC result allowed for the determination that a negative test result was due to a lack of crossreactivity with the assay's oligonucleotides, and not due to an inhibitor of amplification present in a processed sample. One strain of *N. cinerea* (#565) (previously identified as *N. lactamica*) led to the Diplex GC assay's IAC not being detected. The IAC was detected in the assay post a dilution of the sample (1×10^5 cells/SDA reaction). This diluted sample also produced a Negative result for

crossreactivity. Two strains of *Neisseria lactamica* (#23970 and 391) were determined to crossreact with the assay. Additionally, a very weak positive result was detected with 1 out of 3 replicates of *Branhamella catarrhalis* strain #25240. All strains of *Neisseria kochii* were positive with the SDA assay for *Neisseria gonorrhoeae*. This was expected since the organism has been identified as a subspecies of *Neisseria gonorrhoeae.*

[074] These results demonstrate the crossreactivity pattern of the assay described in this invention.

Table 6. *Neisseria gonorrhoeae* strains

Organism	Strain	Source	Result
Neisseria gonorrhoeae	19424	ATCC	Pos.
Neisseria gonorrhoeae	35542	ATCC	Pos.
Neisseria gonorrhoeae	43069	ATCC	Pos.
Neisseria gonorrhoeae	43070	ATCC	Pos.
Neisseria gonorrhoeae	49226	ATCC	Pos.
Neisseria gonorrhoeae	51109	ATCC	Pos.
Neisseria gonorrhoeae	454	BD	Pos.
Neisseria gonorrhoeae	1632	BD	Pos.
Neisseria gonorrhoeae	2900	BD	Pos.
Neisseria gonorrhoeae	111	CDC	Pos.
Neisseria gonorrhoeae	4	BD	Pos.
Neisseria gonorrhoeae	20	BD	Pos.
Neisseria gonorrhoeae	98	BD	Pos.
Neisseria gonorrhoeae	115	BD	Pos.
Neisseria gonorrhoeae	497	BD	Pos.
Neisseria gonorrhoeae	1588	BD	Pos.
Neisseria gonorrhoeae	1618	BD	Pos.
Neisseria gonorrhoeae	2373	BD	Pos.
Neisseria gonorrhoeae	3981	BD	Pos.
Neisseria gonorrhoeae	8000	BD	Pos.
Neisseria gonorrhoeae	8002	BD	Pos.
Cloned *Neisseria gonorrhoeae* DNA (plasmid)	N/A	N/A	Pos.
Negative Control	N/A	N/A	Neg.

Table 7. Neisseria *S*pecies Crossreactants

Organism	Strain/Organism #	Site	Result
Neisseria cinerea	232	BD	Neg.
Neisseria cinerea	565	BD	Neg.
Neisseria elongata	25295	ATCC	Neg.

Neisseria elongata ss nitroreduscens	49377	BD	Neg.
Neisseria elongate ss nitroreduscens	49378	ATCC	Neg.
Neisseria elongata ss glycolytica	29315	ATCC	Neg.
Neisseria flava	nrl 30008	HVMC	Neg.
Neisseria flava	nrl 30034	HVMC	Neg.
Neisseria flava	nrl 30037	HVMC	Neg.
Neisseria flava	nrl 30136	HVMC	Neg.
Neisseria flava	nrl 30137	HVMC	Neg.
Neisseria flavescens	13120	ATCC	Neg.
Neisseria flavescens	13115	ATCC	Neg.
Neisseria flavescens	13116	ATCC	Neg.
Neisseria flavescens	13117	ATCC	Neg.
Neisseria lactamica	264	BD	Neg.
Neisseria lactamica	391	BD	Pos.
Neisseria lactamica	408	BD	Neg.
Neisseria lactamica	803	BD	Neg.
Neisseria lactamica	23970	ATCC	Pos.
Neisseria lactamica	7624	BD	Neg.
Neisseria lactamica	216-75	ATCC	Neg.
Neisseria meningitidis	13077	ATCC	Neg.
Neisseria meningitidis	305	BD	Neg.
Neisseria meningitidis	14685	ATCC	Neg.
Neisseria meningitidis	35699	BD	Neg.
Neisseria meningitidis	41799	BD	Neg.
Neisseria meningitidis	55592	ATCC	Neg.
Neisseria meningitidis	1490	CCF	Neg.
Neisseria meningitidis	2039	CCF	Neg.
Neisseria meningitidis	2040	CCF	Neg.
Neisseria meningitidis	c136	CCF	Neg.
Neisseria meningitidis(768)	301	BD	Neg.
Neisseria meningitidis (LRD728)	53900	BD	Neg.
Neisseria muscosa	19693	ATCC	Neg.
Neisseria muscosa	19694	ATCC	Neg.
Neisseria muscosa	19695	ATCC	Neg.
Neisseria mucosa	19696	ATCC	Neg.
Neisseria muscosa	1915	CCF	Neg.
Neisseria perflava	6573	BD	Neg.
Neisseria perflava	8078	BD	Neg.
Neisseria perflava	8028	BD	Neg.
Neisseria perflava	nrl 30015	HVMC	Neg.
Neisseria perflava	nrl 30035	HVMC	Neg.
Neisseria perflava	nrl 35304	HVMC	Neg.
Neisseria perflava	nrl 35308	HVMC	Neg.
Neisseria perflava	nrl 35320	HVMC	Neg.
Neisseria polysaccharea	1574	CCF	Neg.
Neisseria polysaccharea	43768	ATCC	Neg.
Neisseria sicca	Msdh	BD	Neg.

Neisseria sicca	29256	ATCC	Neg.
Neisseria sicca	29259	ATCC	Neg.
Neisseria sicca	891	CCF	Neg.
Neisseria sicca	9913	ATCC	Neg.
Neisseria subflava	10555	BD	Neg.
Neisseria subflava	7697	BD	Neg.
Neisseria subflava	7858	BD	Neg.
Neisseria subflava	209	CCF	Neg.
Neisseria subflava	616	CCF	Neg.
Neisseria subflava	1253	CCF	Neg.
Neisseria subflava	1378	CCF	Neg.
Neisseria subflava	1889	CCF	Neg.
Neisseria subflava	2007	CCF	Neg.
Neisseria subflava	nh14	Farrell	Neg.
Neisseria subflava	nh15	Farrell	Neg.
Neisseria subflava	nh18	Farrell	Neg.
Neisseria subflava	nh7	Farrell	Neg.
Neisseria subflava	nh8	Farrell	Neg.
Neisseria subflava	nh9	Farrell	Neg.
Neisseria weaveri	1246	CCF	Neg.
Neisseria weaveri	1399	CCF	Neg.
Neisseria weaveri	1862	CCF	Neg.
Neisseria kochii	31291	NRL	Pos.
Neisseria kochii	31292	NRL	Pos.
Neisseria kochii	31294	NRL	Pos.
Neisseria kochii	32895	NRL	Pos.
Neisseria kochii	32896	NRL	Pos.
Negative Control	N/A	N/A	Neg.

Table 8. Non Neisseria Crossreactants

Non Neisseria Crossreactants	**ATCC or ID.**	**Source**	**Result**
Acinetobacter calcoaceticus	13339	ATCC	Neg.
Acinetobacter lwolfi	19001	ATCC	Neg.
Actinomyces israelii	10049	ATCC	Neg.
Aeromonas hydrophilia	7966	ATCC	Neg.
Alcaligenes faecalis	8750	ATCC	Neg.
Bacillus subtilis	12100	ATCC	Neg.
Bacteroides fragilis	25285	ATCC	Neg.
Branhamella catarrhalis	25238	ATCC	Neg.
Branhamella catarrhalis	25240	ATCC	*Weak Pos.
Branhamella catarrhalis	25238	ATCC	Neg.
Branhamella catarrhalis	c223	CCF	Neg.
Branhamella catarrhalis	c224	CCF	Neg.
Branhamella catarrhalis	c225	CCF	Neg.

Candida albicans	44808	ATCC	Neg.
Candida glabrata	2001	ATCC	Neg.
Candida tropicalis	750	ATCC	Neg.
Chlamydia pneumoniae	AR-39	ATCC	Neg.
Chlamydia psittaci	Cal-10	BD	Neg.
Chlamydia trachomatis	LGV II	BD	Neg.
Citrobacter freundii	8090	ATCC	Neg.
Clostridium perfringens	13124	ATCC	Neg.
Corynebacterium renale	19412	ATCC	Neg.
Cryptococcus neoformans	36556	ATCC	Neg.
Edwardsiella tarda	15469	ATCC	Neg.
Enterobacter cloacae	13047	ATCC	Neg.
Enterococcus faecalis	29212	ATCC	Neg.
Enterococcus faecium	19434	ATCC	Neg.
Escherichia coli	11775	ATCC	Neg.
Flavobacterium meningosepticum	13253	ATCC	Neg.
Gardnerella vaginalis	14018	ATCC	Neg.
Gemella haemolysans	10379	ATCC	Neg.
Haemophilus influenzae	33533	ATCC	Neg.
Kingella kingae	23330	ATCC	Neg.
Klebsiella pneumoniae	13883	ATCC	Neg.
Lactobacillus acidophilus	4356	ATCC	Neg.
Lactobacillus brevis	14869	ATCC	Neg.
Lactobacillus jensenii	25258	ATCC	Neg.
Listeria monocytogenes	7644	ATCC	Neg.
Moraxella lacunata	17967	ATCC	Neg.
Moraxella osloensis	9281	BD	Neg.
Morganella morganii	25830	ATCC	Neg.
Peptostreptococcus anaerobius	27337	ATCC	Neg.
Peptostreptococcus asaccharolyticus	29743	ATCC	Neg.
Peptostreptococcus productus	27340	ATCC	Neg.
Plesiomonas shigelloides	14029	ATCC	Neg.
Propionibacterium acnes	6919	ATCC	Neg.
Providencia stuartii	35031	ATCC	Neg.
Pseudomonas aeruginosa	27853	ATCC	Neg.
Salmonella minnesota	9700	ATCC	Neg.
Salmonella typhimurium	13311	ATCC	Neg.
Staphylococcus aureus	25923	ATCC	Neg.
Staphylococcus epidermidis	E155	ATCC	Neg.
Streptococcus agalactiae	12386	ATCC	Neg.
Streptococcus mitis	9811	ATTC	Neg.
Streptococcus mutans	25175	ATCC	Neg.
Streptococcus pneumoniae	6303	ATCC	Neg.
Streptococcus pyogenes	19615	ATCC	Neg.
Streptomyces griseus	10137	ATCC	Neg.

Veillonella parvula	10790	ATCC	Neg.
Vibrio parahaemolyticus	17802	ATCC	Neg.
Yersinia enterocolitica	27729	ATCC	Neg.

Example 5: Demonstration of Diplex SDA Assay for *Neisseria gonorrhoeae*

[075] The following data demonstrates the ability of a diplex SDA assay for *Neisseria gonorrhoeae* to amplify and detect two target DNA sources (*Neisseria gonorrhoeae* target DNA and an IAC, *e.g.*, GCI3-IAC4 (SEQ ID NO:9)). This experiment utilized the primary oligonucleotides in Table 2 and the SDA reaction conditions described in Example 2 (see paragraph 067). The data listed in Table 9 demonstrates that the diplex SDA assay for *Neisseria gonorrhoeae* can amplify and detect, in a single assay well, both *Neisseria gonorrhoeae* DNA (GC) and the internal amplification control (IAC). The two DNA target sources were distinguished by the use of different fluorescently labeled detection probes: rhodamine (ROX) for *Neisseria gonorrhoeae* and fluorescein (FAM) for the IAC. The *Neisseria gonorrhoeae* DNA and the IAC can be amplified and detected when they were present alone (Conditions 1 & 2), indicating the ability of the assay to detect both target DNA and the IAC. The *Neisseria gonorrhoeae* target sequence was also detected in the presence of the IAC (Condition 3). The *Neisseria gonorrhoeae* signal was not significantly altered when IAC was present. The IAC signal was found to be suppressed in Condition 3. However, this does not reflect on the specificity or sensitivity of the assay, as it is important for the IAC to be detected in the absence of *Neisseria gonorrhoeae* to indicate a lack of inhibitors or presence of human error.

Table 9. Dual Detection Experiment

	Condition # 1		**Condition # 2**		**Condition # 3**	
	ROX	**FAM**	**ROX**	**FAM**	**ROX**	**FAM**
GC Target	100	100	0	0	100	100
IAC Target	0	0	300	300	300	300
MOTA:	68060	1220	240	39900	71370	11440
	52860	2580	40	50650	57370	14430
	81610	1760	160	51570	67760	18230
	53710	1400	170	69930	89480	7720

	82700	2410	150	60770	75250	8210
	55320	3000	30	75640	85300	12380
Mean:	**65710**	**2062**	**132**	**58077**	**74422**	**12068**

REFERENCE CITED AND EQUIVALENTS

[076] All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

[077] Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims along with the full scope of equivalents to which such claims are entitled.